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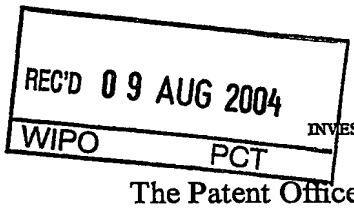

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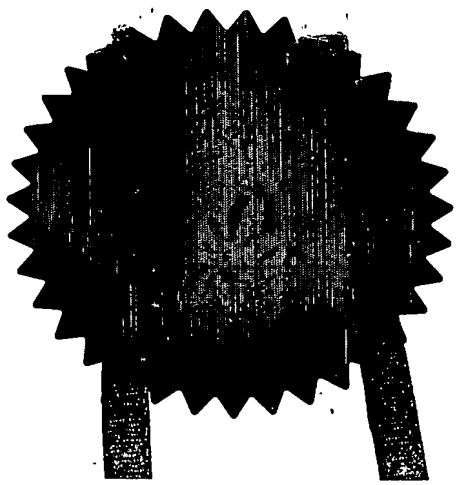

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4. Title of the invention Reversible immortalization of OEG from human olfactory bulbs as a tool to promote spinal cord regeneration

5. Name of your agent (if you have one)
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Reversible immortalization of OEG from human olfactory bulbs as a tool to promote spinal cord regeneration

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TECHNICAL FIELD OF THE INVENTION

The present invention is enclosed within the pharmaceutical research and the industry field. It is focused on the capacity of a specific cell type to promote neuronal regeneration, thus it will be considered in the field of cell therapy.

BACKGROUND OF THE INVENTION:

From the studies of Cajal it is known that the ability of adult CNS neurons to regenerate is extremely limited {Cajal, 1928}. In contrast, neurons from the peripheral nervous system (PNS) have a notable capacity of regeneration, which may be due, among other factors, to the particular characteristics of the Schwann cells (SC) which ensheath PNS axons. This fact has stimulated the use of peripheral nerve and SC grafts to foster regeneration in CNS, with promising results, reviewed in {Jones et all., 2001}). Nevertheless, the incomplete CNS regeneration achieved with SC makes necessary the search for more effective regeneration mediators.

During the last years the use of OEG for CNS regeneration have received plenty of attention, due to their special properties {Raisman 2001}. The olfactory system has remarkable distinctive properties within the adult mammalian central nervous system. Throughout the whole life of an organism, olfactory sensory neurons are renewed from progenitor cells present at the olfactory neuroepithelium (reviewed in {Farbman, 1990} and {Doucette, 1993}). New-born sensory neurons extend new axons that grow and enter in the central nervous system (CNS) to make their appropriate connections in the glomerular layer of the olfactory bulbs. These olfactory axons are surrounded by an

especial type of glial cells, called olfactory ensheathing glia (OEG){Golgi 1875} {Blanes 1898}.

In vivo OEG express several markers that has been used for their identification after isolation and culture (see {Ramon-Cueto and Avila, 1998} and references therein). OEG share some properties with SC and astrocytes (revised in {Doucette, 1990}), although they present a distinct pattern of markers and properties which allows to classify them as a different class of glial cells.

Two OEG variants have been identified in cultures {Pixley 1992}{Ramón-Cueto and Nieto-Sampedro, 1992}{Chuah and Au, 1994}. In serum-free medium Barnett and co-workers have identified two extreme morphological types in the OEG cultures, an astrocyte-like flat cell, which express GFAP (fibrous staining) and PSA-NCAM, and is negative for p75-NGFr, and a second type, a Schwann-like spindle cell, expressing p75-NGFr, with diffuse staining for GFAP and negative for PSA-NCAM. All the intermediate phenotypes are possible and the observation of these phenotypes even in an established clonally cell line supports the view that they can derive from a common precursor {Franceschini and Barnett, 1996}.

In our OEG cultures, from adult rats and human donors, we have also detected both morphological types of cells and the intermediate phenotypes (unpublished observations).

From the data obtained from our lab and other labs, it is obvious that OEGS are clearly distinct from astrocytes or Schwann cells, independently of the age of the murine or human donor.

Promotion of axonal regeneration: in vitro and in vivo

The capacity of OEG to promote neurite outgrowth can be used to establish neuritogenesis and/or regeneration models in culture. Neurite outgrowth in culture may simply indicate neuritogenesis when using embryonic or neonatal neurons. However, neurite extension from adult CNS neurons can be considered as a culture model of CNS regeneration. Thus, Wigley and Berry have established a co-culture model using adult retinal ganglion cells (from now, RGC) on a monolayer of glial cells. Adult RGC cultured on a confluent monolayer of neonatal cortical astrocytes are able to re-grow neurites over long distances {Wigley and Berry, 1988}. In a recent study, Wigley and co-workers, using the same model, compared monolayer of adult rat OEG, with neonatal astrocytes and SC. They demonstrated that adult rat OEG were the best substrate for

neurite regeneration of adult RGC in culture, and this effect mainly depended on intercellular contact and calcium {Sonigra et al., 1999}. Cellular surface molecules (factors for adhesion, axonal guiding, etc.) are important for adult RGC as demonstrated by Sonigra and co-workers, but their exact identity have to be defined.

Initially regeneration in CNS has been achieved by the use of PNS SC grafts (revised in {Jones et al., 2001}). CNS re-growing axons are able to penetrate into this permissive environment but the problem is the low efficiency of re-entry in CNS tissue distal to the lesion. Previous experiments indicated that OEG were able to mediate re-entry of peripheral sensory axons in spinal cord CNS environment after rizhotomy {Ramón-Cueto and Nieto-Sampedro, 1994}. Re-entry of regenerating axons into the CNS has also been observed after a complete transection of the spinal cord, when a bridge tube with SC and OEG was placed. The presence of OEG decreased the formation of glial scar and constituted an axonal passageway at the interface graft-host {Ramón-Cueto et al., 1998}. This method provided a way of increasing the efficiency of re-entry of regenerating axons in the CNS host tissue. Raisman and co-workers had also showed recovery of the corticospinal tract function after OEG grafting in partially transected spinal cord {Li et al., 1997}. Using the model of rat spinal cord complete transection, an extraordinary grade of CNS regeneration affecting to several tracts in the spinal cord, has also been demonstrated, with accompanying functional recovery as measured by behavioural tests {Ramón-Cueto et al., 2000}. OEG seem to be able to migrate in the host CNS tissue and to go along with the growing axons thus providing a micro-environment favourable for CNS regeneration {Li et al., 1998} {Ramón-Cueto et al., 2000}.

All these data, support the viewthat OEGs are functionally different from the other neural cell , such as astrocytes or Schwan cells.

The object of our invention is precisely based on the capacity of OEG to promote neuronal axonal regeneration in vivo, an ability not shared, so far , by any other cell type primary or genetically modified, at this extent.

Limited growth rate.

One of the most important requisites for the use of these cells is a complete understanding of their growth requirements. Certain mitogens for OEG were initially characterised like factors present in astrocyte conditioned medium (ACM), that do not bind to heparin and can be inhibited with antibodies against neuregulin-1 (NRG-1)

{Pollock et al., 1999}. Moreover, semipurified bovine pituitary extract, which is a crude source of several glial mitogens including GGF a NRG-1, is active for OEG {Pollock et al., 1999}.

Whereas experiments performed in rodents suggest that OEG grafts favour functional recovery after spinal cord injuries {Li et al., 1997} {Ramón-Cueto et al., 2000}, these studies have to be extended to primates, where the supraspinal control of motor functions is fundamental to asses functional recovery.

Of the utmost importance in this respect is the availability of an accessible and non-limited source of human OEG for grafting into patients. We have defined conditions for prolonged subculture of adult rat OEGs using a specific mixture of trophic factors, biochemical compounds and medium. This combination also allows the growth of the human OEGs . However, after long-term culture OEG lose their ability to support axonal regeneration from adult neurons although they keep the ability to support axonal extension from young neurons.

The generation of immortalised non-tumorigenic clonal cell lines which retain the axonal regeneration-promoting properties of primary OEG cultures is an obvious alternative to have unlimited amount of OEGs. We have pursued this approach, using and standard procedure to reversible immortalize primary cells and obtaining several immortalized clonal cell lines of OEGs from human donors. We have tested that these cells lines closely resemble primary OEGs in their molecular markers Futhermore, we have found that they maintained their ability to promote axonal regeneration from adult neurons. Some of these cell lines preserve the regeneration capacity of the primary OEGs and they constitute an unlimited supply of human OEGs.

DETAILED DESCRIPTION OF THE INVENTION:

Cell isolation.

Human cells were obtained from *post-mortem* human tissue from adult donors. The tissue belong to male and female adults donors, from the olfactory bulb. The donor's age appears to be none militant for the success of the culture. The tissue was washed with sterile saline solutions (PBS) containing antibiotic and anti-fungi. Then the tissue was subjected to controlled proteolysis with a trypsin solution at 37°C for 20 min. After this time the tissue was mechanically disrupted and cultured in a specifically designed medium for this cell type. This semi-defined medium contain extract from bovine pituitary, forskolin , low amount of foetal calf serum heat –inactivated in combination with Dulbeco's modified medium (DMEM).

This human cells were maintained in this medium and then were characterized as OEG following the general criteria indicated in Ramon Cueto and Avila (1998) and Moreno-Flores at al. (2003).

Figure 1 showed the general morphology of this cell after dissociation and cultured for one week (A) or after five consecutives passages (B)

Figure 2 shows the immunofluorescence of human OEGs stained with antibodies against glial markers, such as GFAP , Neuroligin, S100 and APP (22C11).

Figure 3 Shows the expression of members of the EGF-R family. Cells extract obtained fro human OEGs in different medium were analysed by Western blot with antibodies against Erb B2, Erb B3, and Erb B4. As a loading control tubulin was used. The Figure contain the data from similar experiment with parallel culture medium from established cell lines from rat OEGs. ,

Table 1 summarise the different cell markers analysed from murine OEGs, astrocytes, Schwann cells and oligodendocite-O2 astrociye lineage and our human OEGs

Table I

Marker	Astrocyte-1	O-2A	Schwann	mGE		hGE
				T.A.	T.S.	
O4	-	+	+	+ -	+ -	nd
p75 NGFr	-	-	+	-	+	+
PSA-N-CAM	-	-	-	+	-	n.d.
GFAP	+(f)	- +	+(d)	+(f)	+/-(d)	+(d)
GalC	-	+	+	-	-	n.d.
HNK-1	-	+	+	-	-	n.d.
A2B5	-	+	-	-	-	n.d.
GD3	-/+	+	-	-	-	n.d.
S100	++	n.d.	+	++		++
22C11	+	n.d.	+	++		++
Vim	+	n.d.	+	+		+
Neuroligin	-	n.d.	n.d.	++		++
Erb2	++	n.d.	n.d.	++		+++
Erb3	+++	n.d.	n.d.	+/++		+
Erb 4	+++	n.d.	n.d.	+/-		+
3-PGDH	n.d.	n.d.	n.d.	++		++

(f) Fibrous staining ; (d): Diffuse staining, n.d. not determined

To immortalize these human OEGs we used a reversible system, described by Dr. D. Trono's lab (Naldini et al., 1996; Salmon el al., 2000), using the lentiviral constructs, denoted by pH Lox-CMV-Ttag-IRES-TK and pH Lox-CMV-HTERT-IRES-TK (MTA forms-Trono Lab)

From these infections, established clones were obtained following those human cells with the highest growth rate; and initially, some clones have been obtained and characterized as OEGs.

For instance the cells immortalized with pH Lox-CMV-Ttag-IRES-TK, expressed T antigen (>85%), S100 and 3-PDGH.

Adult Retinal axonal regeneration assay.

The promotion of axonal regeneration from adult rat retinal ganglion neurons (RGN) is used as a standard procedure determining regeneration capacity in cell culture. This method is that reflect the in vivo reparative properties of OEGs, more closely than other neuritogenic assays.

The method used was as follow: retinas were dissected from P60 rat eyes, dissociated and digested with 0.1% trypsin. Digestion was stopped with 2 mg/ml of soybean inhibitor of trypsin, and a fine suspension of cells was obtained by passing the digested material through Pasteur pipettes of decreasing diameters. Cells were centrifuged and resuspended in a defined medium (Moreno-Flores et al., 2003). RGN were plated on monolayers of glial cells: primary OEG or in the establilshed cell lines.

For immunocytochemistry , neuronal- human OEG mixed culture were fixed with 4% paraformaldehyde, after seven days of culture.

Figure 4 shows the axonal regeneration capacity of rat retinal neurons stained with antibodies against neuronal proteins such as neuronal specific tubulin (334), axonal proteins such as MAP1B-SMI31 or MAP2 (305), o somato-dendritic protein MAP2 (514).

Our data shows that human OEG promoted axonal regeneration from adult RGN to a similar extent than either primary OEGs or clonal cell lines from rat OEGs.

Considering that several recent studies have demonstrated the capacity of OEG to promote *in vivo* CNS axonal regeneration (Li et al. 1997 and 1998, Ramón-Cueto et al. 1998 and 2000), with impressive motor and sensory functional recovery even after complete transection of the spinal cord (Ramon-Cueto et al. 2000). and that our recent preliminary experiments shows that established cell lines from rat source maintained such neuronal regenerative capacities in culture(Moreno-Flores et al 2003) and in vivo(Moreno-Flores et al unpublished data), we conclude that the immortalization of this

specific cell type did not produce a significant reduction in the regenerative capacity, even though the molecular mechanism underlined are not known so far.

To our knowledge this is the first time that it has been demonstrated that immortalised human OEGs are able to promote axonal regeneration of cultured neurons from mature mammalian CNS. Our human OEG lines represent useful tools to advance the use of OEG in both research and application:

Thus we propose that this cell lines and similar ones, alone or in pharmaceutical compositions comprising same, may be used to treat CNS lesions

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Claims

1. An immortalised olfactory ensheathing glia cell line.

2. A cell line according to claim 1, for use in a method of therapy.

3. A cell line according to claim 2, for use in promoting neuronal regeneration.

4. A cell line according to any preceding claim, which is a human cell line.

5. A pharmaceutical composition comprising a cell line of any preceding claim, and a pharmaceutically acceptable carrier.

ABSTRACT

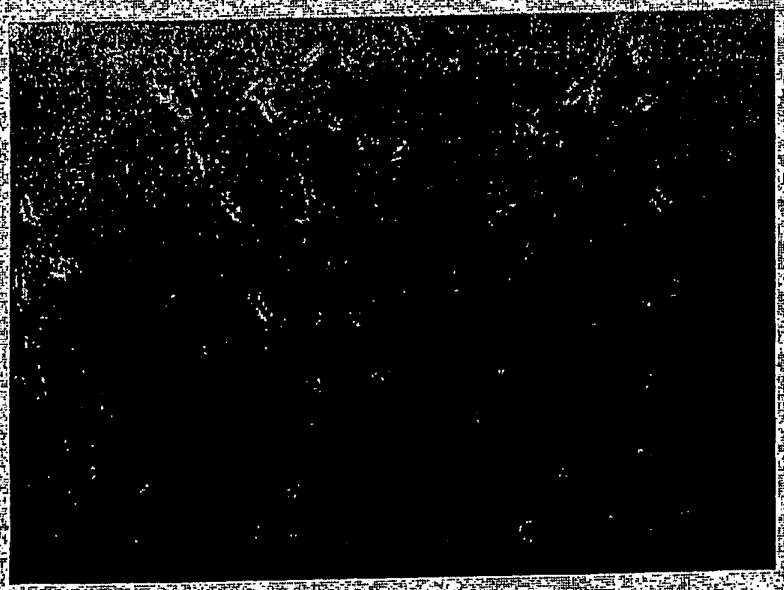
The present invention is based on the capacity of the Olfactory Ensheathing Glia (*from now* OEG) to foster axonal regeneration in the adult mammalian central nervous system (CNS). This specific capacity is probably due to a combination of several factors, such as the molecular composition of cellular membrane and/or the capacity to secrete some molecules; combined with the capacity to reduce glial scar and accompany new growing axon in the damaged CNS.

We have developed immortalized cell lines from primary human OEGs. The cells were cultured from *post-mortem* human tissue from donors and immortalized using a reversible system. These OEG human clones promoted axonal regeneration from adult rat retinal ganglion neurons in a similar fashion to primary OEGs. Considering that this is the first characterization of the ability to stimulate regeneration of such cell type isolated from human olfactory bulb, we propose that these cell lines, alone or in pharmaceutical compositions comprising these cells, may be used to repair lesions in the CNS.

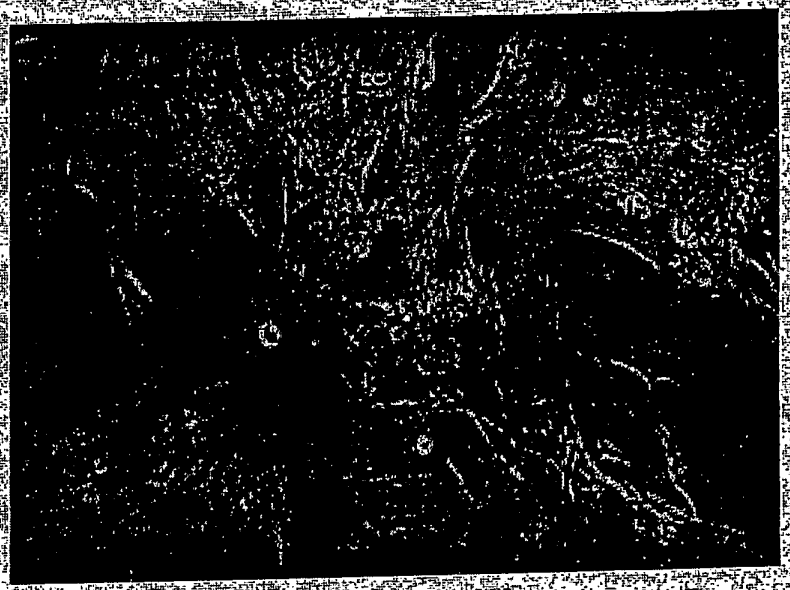
Fig. 1

FIGURE 1

HUMAN OEGs



Human OEGS
DIV 1

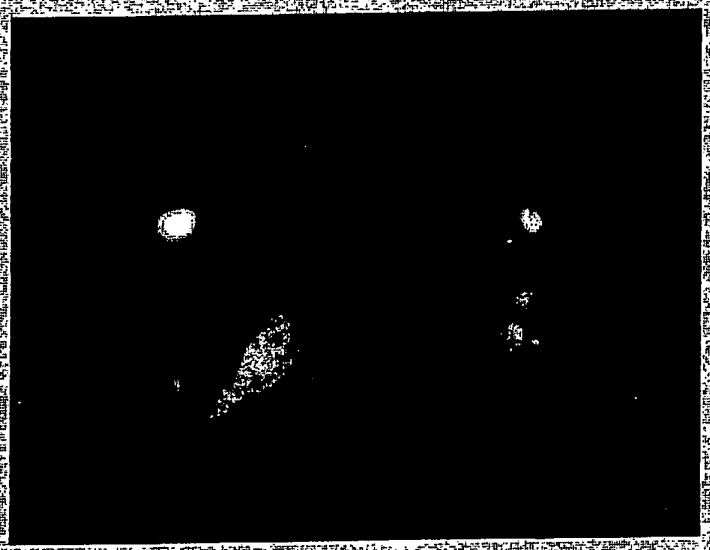


Human OEGS
DIV 5

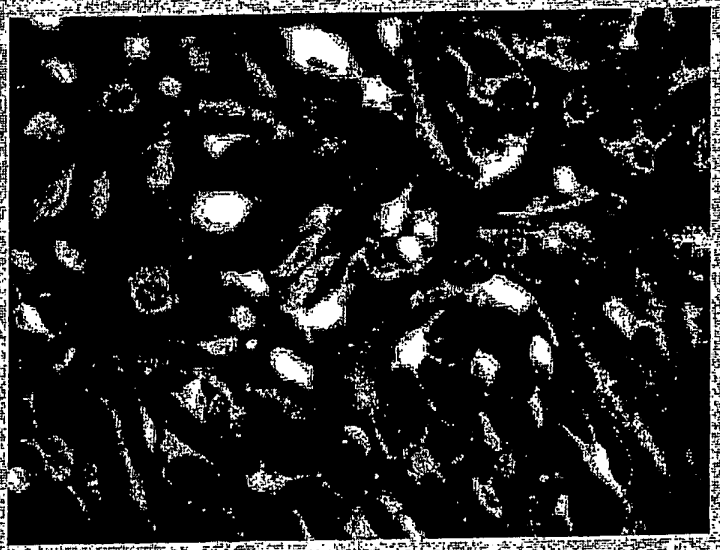
(Staining for beta-galactosidase specific of senencence)

FIGURE 2

HUMAN OEGs



Neuroligin



22C11

S100

GFAP

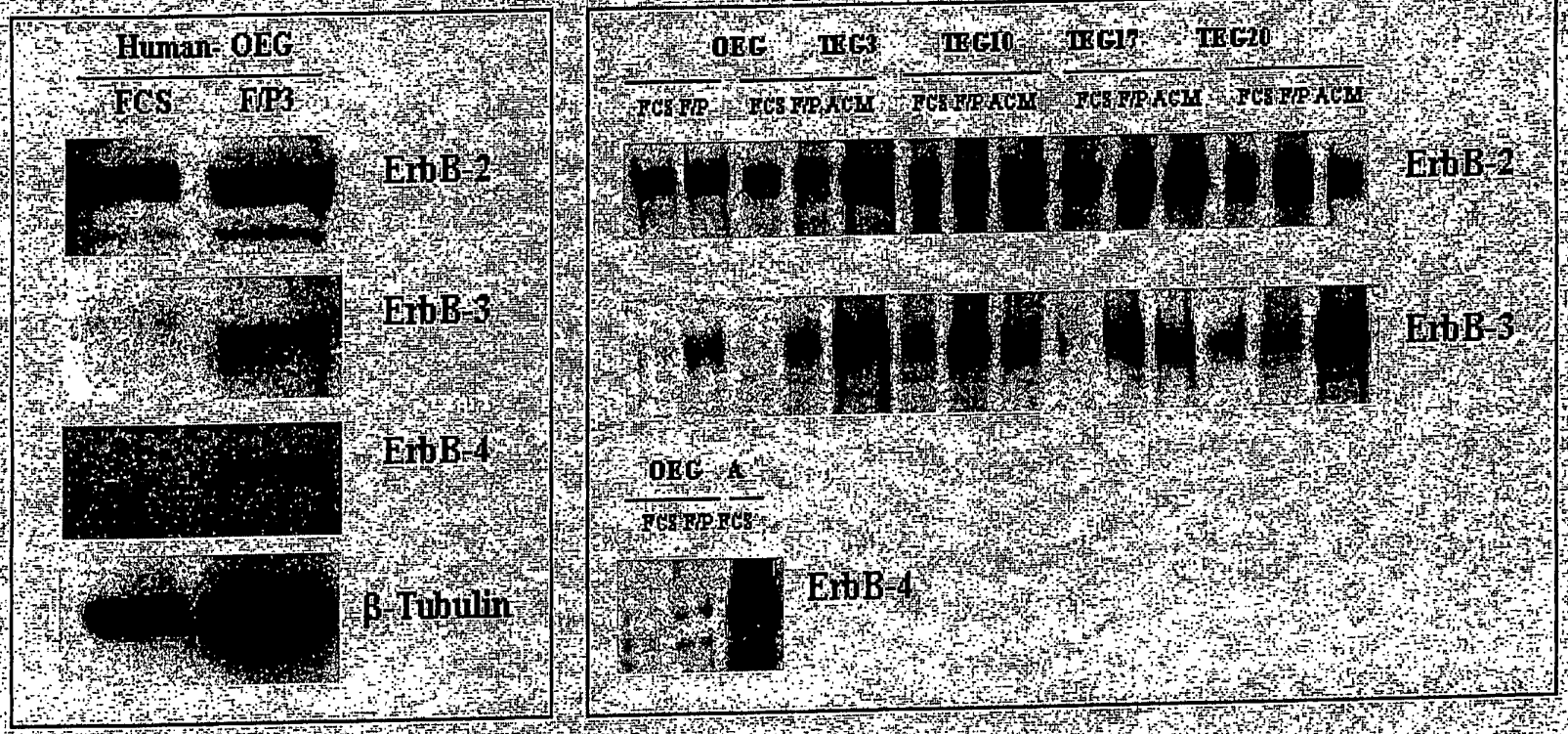
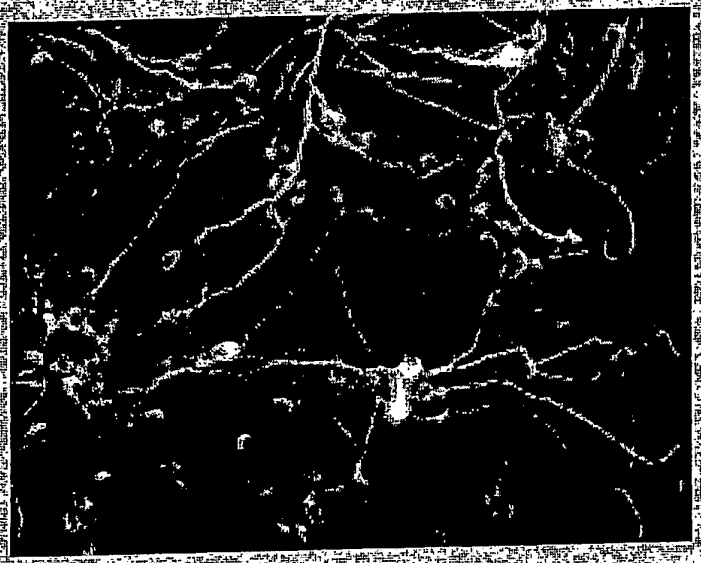

Human OEG
FCS
F/P3
ErbB-2
ErbB-3
ErbB-4
β-Tubulin
OEG
TEG3
TEG10
TEG17
TEG20
FCS F/P
FCS F/P ACM
FCS F/P ACM
FCS F/P ACM
FCS F/P ACM
ErbB-2
ErbB-3
OEG
A
FCS F/P FCS
ErbB-4

FIGURE 3

FIGURE 4

HUMAN OEGs-NGR



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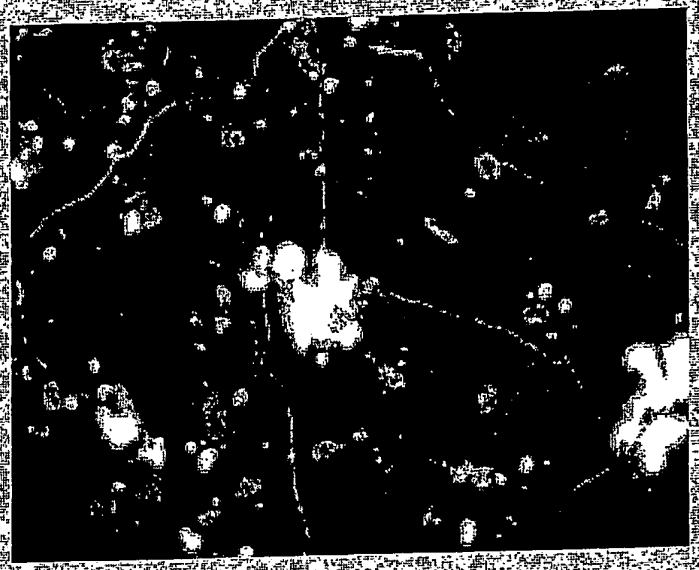

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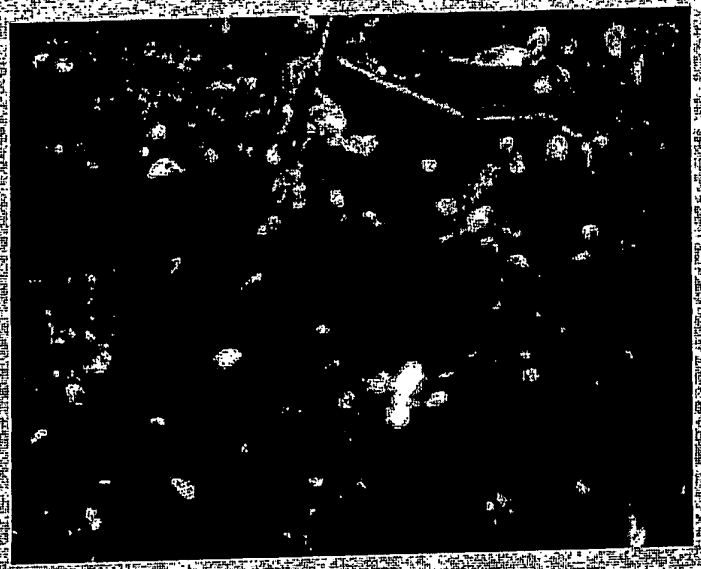

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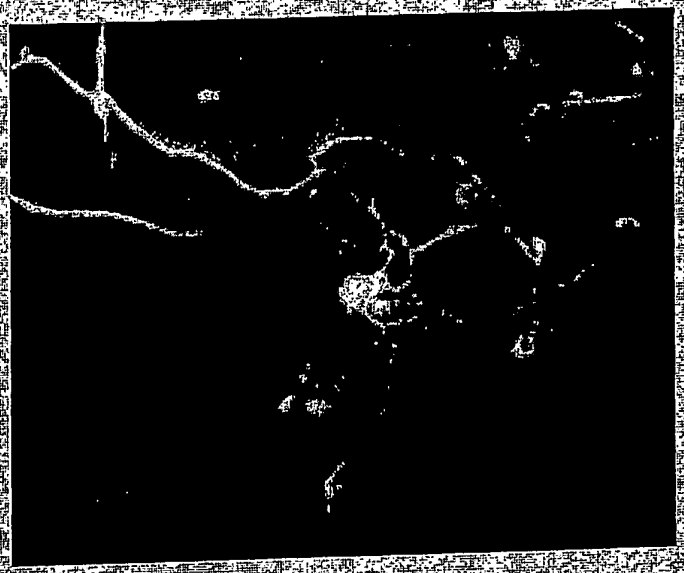

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